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RESEARCH ARTICLE

MAGEA1 inhibits the expression of BORIS via increased promoter methylation

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ABSTRACT

Melanoma-associated antigen A1 (MAGEA1) and BORIS (also known as CTCFL) are members of the cancer testis antigen (CTA) family. Their functions and expression-regulation mechanisms are not fully understood. In this study, we reveal new functions and regulatory mechanisms of MAGEA1 and BORIS in breast cancer cells, which we investigated in parental and genetically manipulated breast cancer cells via gene overexpression or siRNA-mediated downregulation. We identified the interaction between MAGEA1 and CTCF, which is required for the binding of MAGEA1 to the *BORIS* promoter and is critical for the recruitment of DNMT3a. A protein complex containing MAGEA1, CTCF and DNMT3a was formed before or after conjunction with the BORIS promoter. The binding of this complex to the BORIS promoter accounts for the hypermethylation and repression of BORIS expression, which results in cell death in the breast cancer cell lines tested. Multiple approaches were employed, including co-immunoprecipitation, glutathione S-transferase pull-down assay, co-localization and cell death analyses using annexin V-FITC/propidium iodide double-staining and caspase 3 activation assays, chromatin immunoprecipitation and bisulfite sequencing PCR assays for methylation. Our results have implications for the development of strategies in CTA-based immune therapeutics.

KEY WORDS: MAGEA1, CTCF, BORIS, Apoptosis, Methylation

INTRODUCTION

Cancer testis antigens (CTAs) have been extensively studied in a variety of cancers of diverse histological origin and germinal cells (Lim et al., 2012; Zajac et al., 2017). However, the majority of these studies focused on the correlative expression data and/or on evaluating their immunotherapeutic targeting or tumor biomarker values, with limited studies on their gene expression regulation and even fewer on their cellular function in cancer cells (Lim et al., 2012; Kulkarni and Uversky, 2017).

It is generally accepted that their expression is controlled by epigenetics and by methylation in particular (Lim et al., 2012; Zajac et al., 2017; Adair and Hogan, 2009; Schwarzenbach et al., 2014). In addition, the gene regulation among different CTAs has been reported in several cases, including the methylation-independent transcriptional regulation of TSP50 (a CTA; also known as PRSS50) by the brother of the regulator of imprinted sites (BORIS; also known as CTCFL) (Zajac et al., 2017) and BORIS-activated and methylation-dependent MAGEA1 expression (Schwarzenbach et al., 2014; Vatolin et al., 2005; Loukinov et al., 2006). However, expression of MAGEA1 occurs in the absence of BORIS activation, suggesting that BORIS is not an obligate factor for activation of MAGEA1 in melanoma (Kholmanskikh et al., 2008). Contradictory and opposing effects (both tumor promoting and suppressing) have been reported for these CTAs and their related genes/proteins, including CTCF (Salmaninejad et al., 2016). MAGEA1 promotes melanoma proliferation and migration through C-JUN (also known as JUN) activation (Wang et al., 2016), but MAGEA4 interacts with the liver oncoprotein gankyrin and suppresses its tumorigenic activity (Nagao et al., 2003). We have recently shown that MAGEA1 inhibits cell proliferation and migration in breast and ovarian cancer cells via its interaction with FBXW7 and regulates ubiquitin ligase-mediated turnover of NICD1 (Zhao et al., 2017). Similarly, the role of BORIS in cancer is not clearly defined. Although it has been hypothesized and demonstrated to exhibit oncogenic properties (Horibe et al., 2017; Tiffen et al., 2013; Zampieri et al., 2014), its inhibitory effects have also been reported (Tiffen et al., 2013). CTCF, however, has been postulated to be a candidate tumor suppressor (Tiffen et al., 2013).

BORIS is a complex and highly versatile transcription factor (Kalejs and Erenpreisa, 2005; Klenova et al., 2002) and has been shown to activate MAGEA expression via demethylation in various cell types (Kang et al., 2007; Vatolin et al., 2005). Aberrant BORIS expression has been detected in 70% of all primary tumors and cancer cell lines (D'Arcy et al., 2008; Hong et al., 2005; Pugacheva et al., 2010; Smith et al., 2009; Tiffen et al., 2013; Vatolin et al., 2005). In addition, BORIS has been implicated in numerous regulatory functions (Klenova et al., 2002; Monk et al., 2008; Suzuki et al., 2010). BORIS has been shown to be mainly regulated by promoter methylation modification (Hoffmann et al., 2006; Loukinov et al., 2002; Woloszynska-Read et al., 2007), and hypomethylation results in the universal expression of BORIS in cancer cells (Renaud et al., 2007; Woloszynska-Read et al., 2007, 2011). The expression of BORIS is suppressed by CTCF binding to its promoter and enhanced promoter methylation (Renaud et al., 2007). *CTCF* is the only paralog of the *BORIS* gene, sharing a central 11-zinc-finger DNA-binding domain, but having distinct amino and carboxy termini (D'Arcy et al., 2008; Hore et al., 2008; Klenova et al., 2002; Loukinov et al., 2002). In contrast to BORIS, which has restricted expression in testis among normal tissues, CTCF is ubiquitously expressed and often displays opposite functions to those of BORIS (Barski et al., 2007; Heath et al., 2008; Hore et al., 2008; Kim et al., 2007; Lobanenkov et al., 1990; Loukinov et al., 2002).

These published data suggest that the regulation and functions of these CTAs are rather complex and can be cancer type, cell line and/or context dependent. As these CTAs are targets for tumor immunotherapy, it is important to conduct more studies on the

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regulatory mechanisms of their aberrant expression and cellular functions in cancers. Our current study focuses on the functions and aberrant gene regulation of MAGEA1 and BORIS, two CTA family members, in breast cancer cells. Functional and in-depth mechanistic studies were performed using multiple approaches. These included examination of the functions and cellular locations of the endogenous and genetically manipulated genes involved via cell death analyses using the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining method and caspase 3 activation, co-immunoprecipitation (co-IP), glutathione S-transferase (GST) pull-down, co-localization, chromatin immunoprecipitation (ChIP) and methylation analyses.

RESULTS

MAGEA1 inhibits BORIS expression in various breast and ovarian cancer cell lines

We have found that several breast cancer cell lines, such as MCF-7, MDA-MB-231 and MDA-MB-468 express low or non-detectable MAGEA1, and SKOV3 cells express a relatively high level of MAGEA1 (Zhao et al., 2017; Fig. 1). As BORIS, another CTA member, has been shown to be a positive regulator of MAGEA1 expression (Kang et al., 2007; Schwarzenbach et al., 2014; Vatolin et al., 2005), we examined BORIS expression levels in breast cancer cells (MCF-7 and MDA-MB-231), in comparison to non-cancerous breast cells (MCF-10A). As expected, the expression of BORIS was not detectable in non-cancerous MCF10A cells, because BORIS is a CTA protein, but it was expressed at reactively high levels in breast cancer cells (MCF-7 and MDA-MB-231) (Fig. S1A,B). Unexpectedly, and in contrast to the positive relationship between *BORIS* and *MAGEA1* expression previously reported (Schwarzenbach et al., 2014; Kang et al., 2007; Vatolin et al., 2005), the expression of the two genes appeared to be negatively correlated in the cell lines tested (Fig. 1A). To determine their functional relationship, we overexpressed or downregulated [using small interfering RNA (siRNA)] MAGEA1 in cells. MAGEA1 overexpression significantly inhibited BORIS expression in MCF-7 and MDA-MB-231 cell lines (Fig. 1B), and MAGEA1 downregulation (Fig. S2A) enhanced BORIS expression in SKOV3 cells (Fig. 1C). Protein expression levels in control and MAGEA1 genetically manipulated cells also showed that MAGEA1 expression had an inhibitory effect on BORIS expression (Fig. 1D). These data revealed a previously unreported regulation of BORIS expression by MAGEA1.

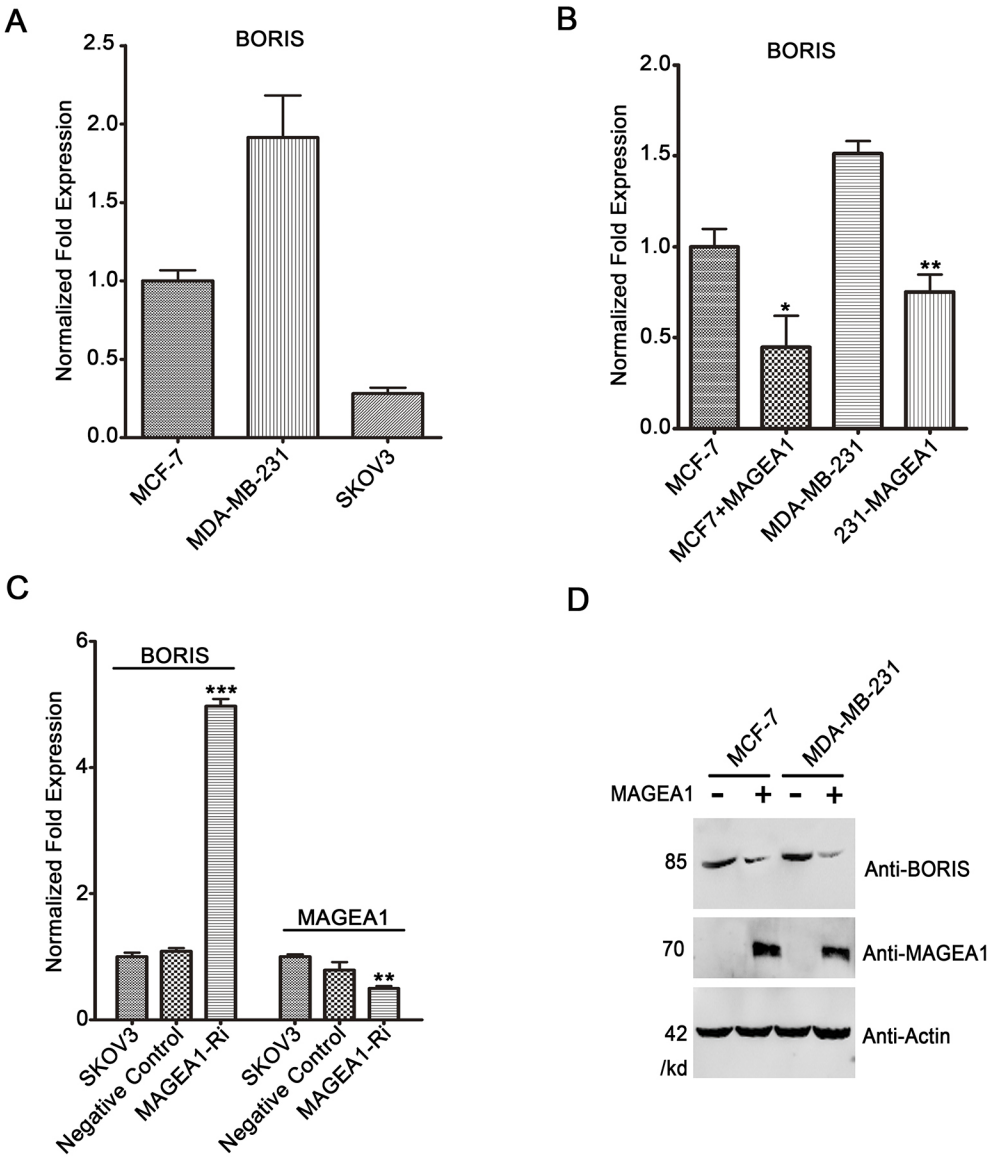

Fig. 1. The inhibitory effects of MAGEA1 on expression of BORIS in breast and ovarian cancer cells. (A) *BORIS* mRNA expression levels in MCF-7, MDA-MB-231 and SKOV3 cell lines, analyzed by qPCR in A to C. GAPDH was used as an internal standard to normalize the expression levels. Statistical analysis of $-\Delta\Delta CT$ values relative to MCF-7 in each group was performed. (B,C) Effects of MAGEA1 overexpression (B) and knockdown (C) on *BORIS* expression in MCF-7, MDA-MB-231 and SKOV3 cells. (D) The expression levels of BORIS protein in the control and MAGEA1-overexpressing cell lines as detected by western blot analyses. All experiments were repeated at least three times. **P*<0.05; ***P*<0.01; ****P*<0.001.

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BORIS is involved in MAGEA1-induced cell death

To determine the functional consequences of BORIS and MAGEA1 expression, we focused on apoptotic cell death. Using flow cytometry analyses, we found that MAGEA1 overexpression induced a significant increase in apoptotic (revealed by the percentage of the annexin V-positive cells on the right side of the *x*-axis) and necrotic (detected by the percentage of the PI-positive cells on the top side of the *y*-axis) cell death from 2.25–5.97% total annexin V-positive cells in the control (parental and vector-transfected) cell lines to ∼21.14–22.09% annexin V-positive cells in MAGEA1-overexpressing MCF-7 and MDA-MB-231 cell lines (Fig. 2A–D). In addition, MAGEA1 overexpression in MCF-7 and MDA-MB-231 cell lines activated caspase 3, revealed by the detection of cleaved caspase 3 (Fig. 2E,F).

To determine the effect of BORIS expression on cell death, we first downregulated BORIS in MCF-7 and MDA-MB-231 cell lines. Reduced BORIS expression resulted in increased cell death, similar to that induced by MAGEA1 overexpression (Figs S2B and S3A,B). On the other hand, BORIS overexpression reduced cell death (both apoptotic and necrotic) in MCF-7 and MDA-MB-231 cells (Fig. 2C,D, upper rows). To test whether the MAGEA1-induced cell death was mediated by BORIS, we overexpressed BORIS in MAGEA1-overexpressing cells. BORIS partially reversed the cell death induced by MAGEA1 (Fig. 2C,D, lower rows). The effects were further demonstrated in the annexin V and PI double-positive cell population (from ∼26.0% to 15.6% in MCF-7 cells, or 21.7% to 11.21% in MDA-MB-231 cells) (Fig. 2C,D), suggesting that the cell death mechanisms may be complex. When BORIS was

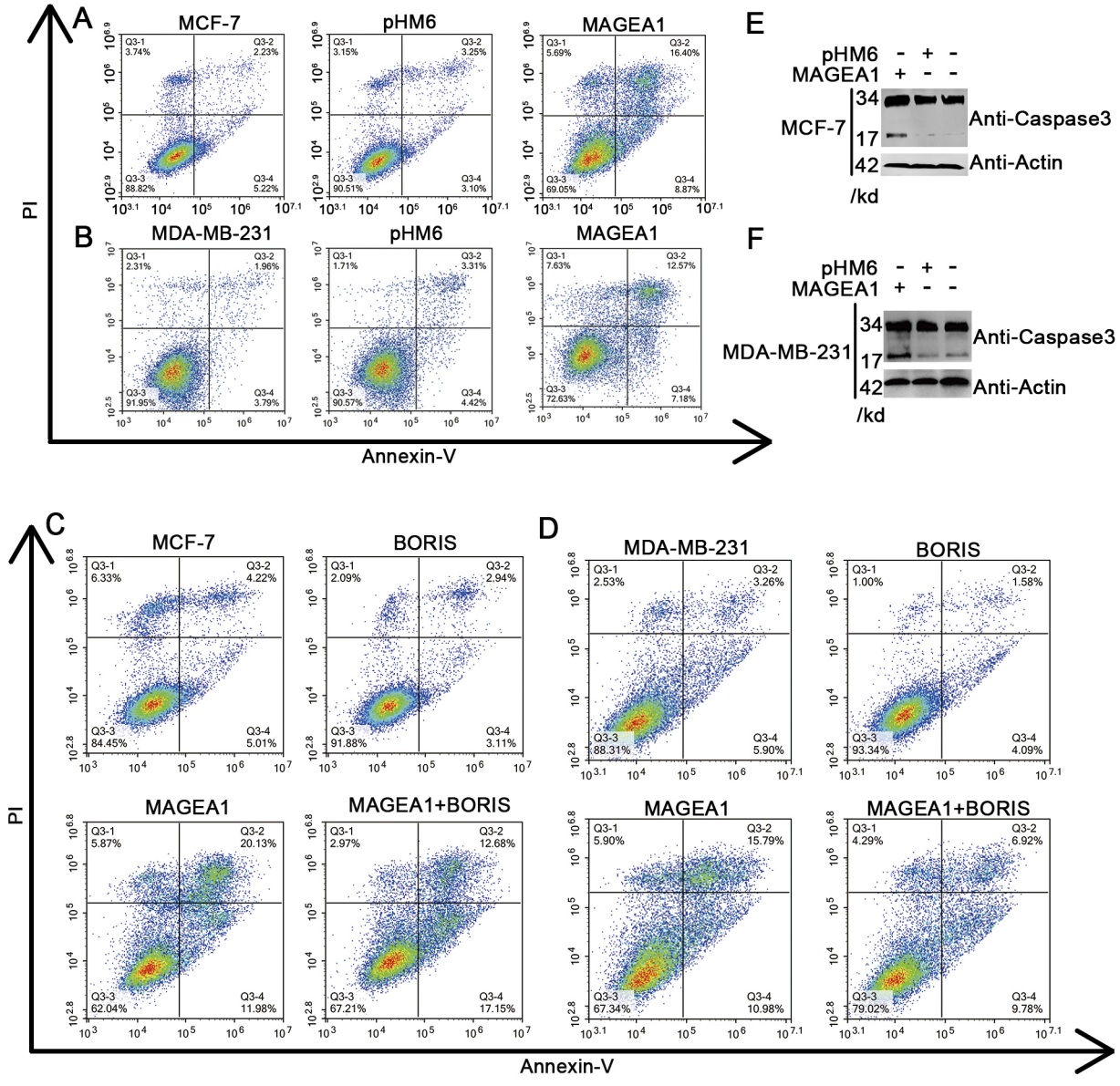

Fig. 2. BORIS reversed the inducing effects of MAGEA1 on apoptosis in breast cancer cells. (A,B) Annexin V-FITC/PI staining detected by flow cytometry analyses in control MCF-7, MDA-MB-231 (pHM6 empty vector plasmid transfected) and their MAGEA1-overexpressing cells. (C,D) Apoptosis levels were detected in MCF-7 (C), MDA-MB-231 (D) and their derived cells: wild-type cells, BORIS-overexpressing cells, MAGEA1-overexpressing cells, and MAGEA1 and BORIS co-overexpressing cells. (E,F) Caspase 3 activation was detected in MCF-7 (E) and MDA-MB-231 (F) parental and MAGEA1-overexpressing cells by western blotting. All experiments were repeated at least three times.

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downregulated, the annexin V-positive, PI-positive and double-positive cell populations were increased (Fig. S3), supporting the promoting effects of BORIS on cell survival.

CTCF was required for MAGEA1-induced suppression of BORIS expression

Because CTCF is a known repressor of BORIS expression (Renaud et al., 2007), we tested whether CTCF was involved in MAGEA1-induced downregulation of BORIS. As expected, downregulation of CTCF and upregulation of MAGEA1 increased and decreased BORIS expression, respectively, in both MDA-MB-231 and MCF-7 cells (Fig. 3A–C). When CTCF was downregulated in MAGEA1-overexpressing cells, it completely reversed the inhibitory effect of MAGEA1 on BORIS expression, suggesting that CTCF is necessary for this effect (Fig. 3A–C). The requirement for CTCF was not mediated by a potential regulatory role of MAGEA1 in CTCF expression, as we found that CTCF expression was not significantly changed at the mRNA and protein levels when MAGEA1 expression was altered in MCF-7, MDA-MB-231 and SKOV3 cells (Fig. S4A–D).

To elucidate the mechanisms of the CTCF requirement, we tested whether MAGEA1 and CTCF interact with each other. The interaction between these two proteins was first detected by co-IP in MCF-7 cells using either an anti-hemagglutinin (HA) (Fig. 3D) or anti-GFP antibody (Fig. 3E) to pull down the HA-tagged MAGEA1 or GFP-fused CTCF proteins and their interacting partner, which was detected by both antibodies in western blots. This interaction was confirmed using GST pull-down assays (Fig. 3F). Importantly, we showed that endogenous MAGEA1 in CTCF proteins interacted with each other in SKOV3 cells, where both proteins were expressed endogenously at significant levels (Fig. 3G). Moreover, both MAGEA1 and CTCF were localized and co-localized in nuclei, consistent with interaction and transcription factor activities (Fig. 3H).

Compared with MAGEA1 overexpression, CTCF downregulation had a relatively weak effect on cell death detected by PI- and annexin V-positive cells (Fig. 4A,B). This effect was likely to be MAGEA1 independent, because MCF-7 and MDA-MB-231 cells did not express detectable endogenous MAGEA1 (Zhao et al., 2017; Fig. 1). However, CTCF downregulation strongly reduced cell death induced by overexpression of MAGEA1 in these cells (Fig. 4A,B), suggesting that CTCF is the critical factor mediating the effects of MAGEA1 on cell death.

CTCF is required for the enhanced methylation induced by MAGEA1 on the BORIS promoter

We conducted ChIP analyses and showed that both MAGEA1 and CTCF bound to the promoter of BORIS (Fig. 5A,B; Fig. S5A–C). The binding of MAGEA1 to the promoter of BORIS was significantly affected by downregulation of CTCF using siRNA-mediated RNA interference (Fig. 5C,D; Fig. S5D,E), suggesting that CTCF is important for the binding ability of MAGEA1 to the BORIS promoter.

MAGEA1 overexpression greatly increased methylation of the BORIS promoter, as detected by bisulfite sequencing, in MCF-7 and MDA-MB-231 cells (Fig. 6A–D). This enhancement activity was significantly reduced when CTCF was downregulated by siRNA interference in MCF-7 cells (Fig. 6E). In addition, the endogenous methylation level of the BORIS promoter was high in SKOV3 cells, where MAGEA1 was relatively highly expressed (Fig. 6F), although the endogenous CTCF expression levels in MCF-7, MDA-MB-231 and SKOV3 cells were comparable (Fig. S4D). These data suggest that MAGEA1 is the driving force for the methylation of the BORIS promoter, which requires CTCF. However, CTCF expression in the absence of MAGEA1 in MCF-7 and MDA-MB-231 cells (Fig. S4D) is insufficient to induce extensive methylation in the BORIS promoter.

DNMT3a participates in the formation of the MAGEA1–CTCF–DNMT3a complex

DNMT3a, an enzyme that catalyzes the transfer of methyl groups to specific CpG structures in DNA, was found to interact with both MAGEA1 and CTCF by GST pull-down assay in MCF-7 cells (Fig. 7A). When CTCF was downregulated, the interaction between MAGEA1 and DNMT3a was significantly decreased (Fig. 7B), suggesting that CTCF is important for the tri-interaction. Immunofluorescence experiments further confirmed that these proteins, MAGEA1 or CTCF and DNMT3a, were co-localized in nuclei in MCF-7 cells (Fig. 7C). On the other hand, MAGEA1 overexpression did not affect the interaction between CTCF and DNMT3a (Fig. 7D).

We tested the role of another DNA methyltransferase, DNMT1. When DNMT1 expression was downregulated by siRNA interference in breast cancer cells, MAGEA1 expression was increased, similar to that reported in melanoma cells (Cannuyer et al., 2013). Under these conditions, BORIS expression was repressed (Fig. S6A,B), consistent with our MAGEA1 overexpression data shown in Fig. 1B. Moreover, MAGEA1 overexpression and DNMT1 knockdown both induced cell apoptosis (Fig. S6C). However, unlike DNMT3a, DNMT1 was not shown to interact with MAGEA1 and CTCF in MCF-7 cells by GST pull-down assays (Fig. 7E), suggesting that the MAGEA1–DNMT3a interaction is specific.

CTCF recruits DNMT3a to the promoter of BORIS, while MAGEA1 enhances the promoter methylation of BORIS by DNMT3a

To investigate whether DNMT3a was functionally involved in the inhibition of BORIS expression by MAGEA1, we performed ChIP assays in MCF-7 cell lines. Downregulation of CTCF resulted in reduced binding of DNMT3a to the promoter of BORIS, independent of MAGEA1 expression (Fig. 8A). Bisulfite sequencing PCR assays revealed that downregulation of DNMT3a resulted in reduced methylation of the BORIS promoter, even when MAGEA1 was overexpressed (Fig. 8B–D). In addition, we tested the functional consequences of downregulation of DNMT3a in cell death induced by MAGEA1 overexpression in MCF-7 and MDA-MB-231 cell lines. As shown in Fig. 8E and F, reduced DNMT3a expression reversed a significant portion of the MAGEA1-induced cell death.

On the other hand, knockdown of DNMT1 did not significantly affect the methylation of the BORIS promoter in the absence and presence of MAGEA1 overexpression (Fig. S7A–C; compare Fig. S7C with Fig. 8B), suggesting that DNMT1 is not functionally involved in this activity.

Our collective data presented in this study demonstrate that the negative regulatory effect of MAGEA1 on cell survival was mediated by its repressive regulatory effect on BORIS expression in the breast cancer cell lines tested. CTCF was required for MAGEA1 to bind to the promoter of BORIS and for the recruitment of DNMT3a for enhanced methylation in the promoter region. DNMT3a, but not DNMT1, was likely the major methyltransferase responsible for DNA methylation and repression of BORIS expression. CTCF and DNMT3a were also functionally involved in MAGEA1-induced cell death.

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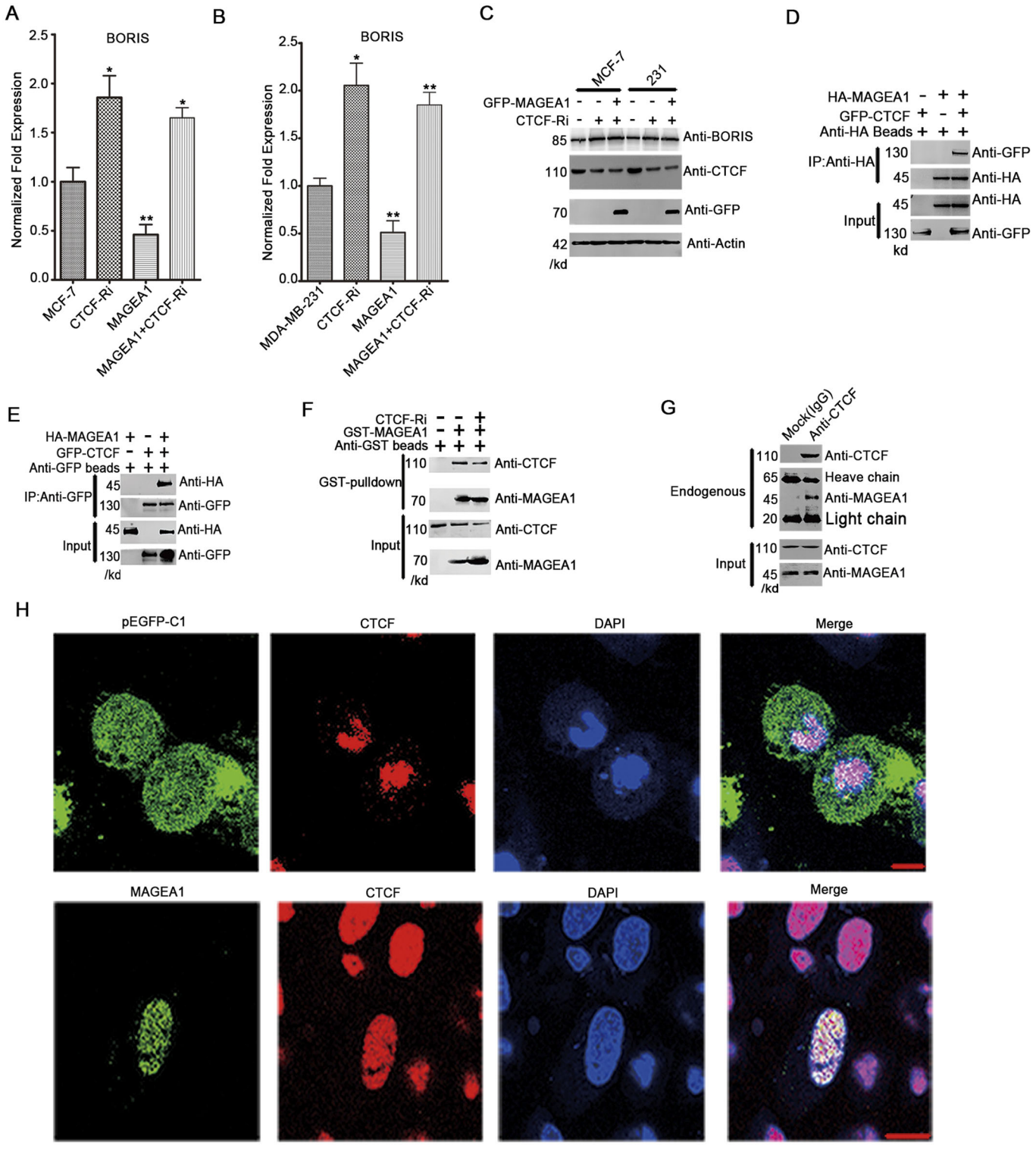

Fig. 3. CTCF is functionally involved in the inhibition of BORIS by MAGEA1. (A,B) MAGEA1 cannot inhibit BORIS expression under the condition of CTCF knockdown in MCF-7 and MDA-MB-231 cells analyzed by qPCR. (C) Western blot assay confirmed that MAGEA1 cannot inhibit BORIS expression without CTCF. (D,E) The interaction between MAGEA1 and CTCF was identified in MCF-7 cells. The expression levels of the proteins encoded by the input genes (transfected GFP-tagged CTCF, HA-tagged MAGEA1) were confirmed by western blot analyses. The incubated proteins are indicated. The antibodies used in the immunoprecipitation (IP) or western blot steps are labeled on each row. (F) GST pull-down assay confirmed the interaction between MAGEA1 and CTCF in MCF-7 cells. All of the incubated proteins and antibodies used in the IP and western blot steps are indicated. (G) Co-IP assays were conducted using anti-CTCF antibodies for endogenous proteins in SKOV3 cells. The antibodies used in the IP step are indicated at the top, with the protein G magnetic bead linked to rabbit IgG as the control. The antibodies used in the western blot step are labeled on the right. (H) The subcellular localizations of MAGEA1 and CTCF were identified by immunofluorescence co-localization assays in MCF-7 cells. MAGEA1 fused to GFP showed green fluorescence, and CTCF was detected by TRITC red fluorescence. Nuclei were stained by DAPI (blue fluorescence). Scale bars: 50 µm. All experiments were repeated at least three times. **P*<0.05; ***P*<0.01.

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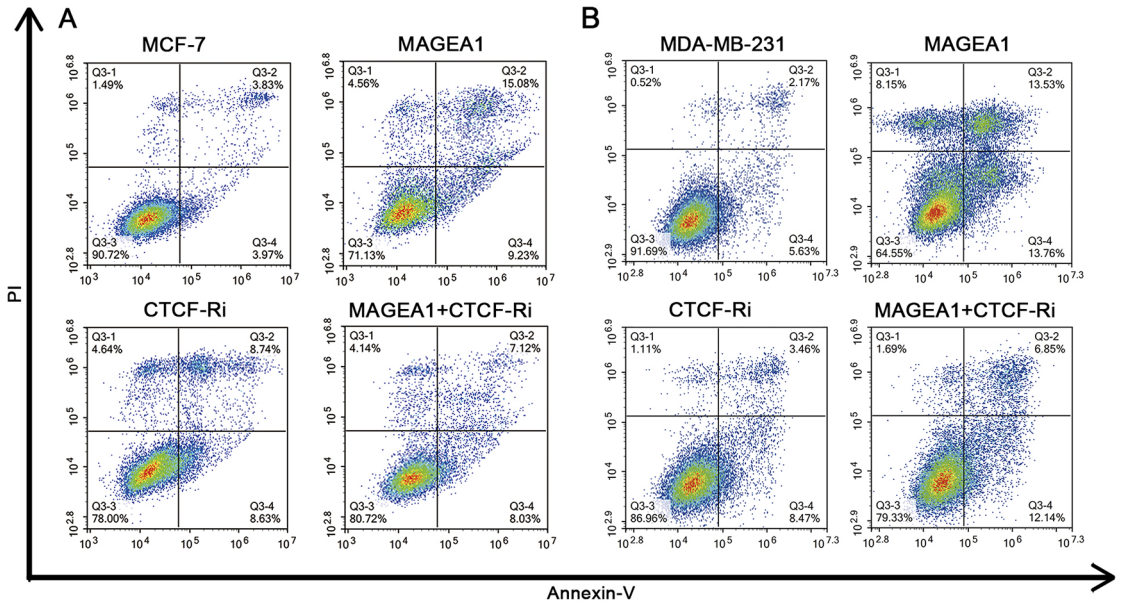

Fig. 4. Downregulation of CTCF reversed cell death induced by MAGEA1. (A,B) Annexin V-FITC/PI staining-based flow cytometry analyses detected the cell death (both apoptotic and necrotic) in MCF-7, MDA-MB-231 and their derived cell lines, including MAGEA1-overexpressing cells, CTCF siRNA interference cells, and MAGEA1-overexpressing and CTCF siRNA interference cells. All experiments were repeated at least three times.

DISCUSSION

To make CTAs effective and practical targets for tumor immunotherapy, it is important to conduct more studies on the regulatory mechanisms of their aberrant expression and cellular functions in cancers. The published data, as reviewed in the Introduction, suggest that the regulation and functions of these CTAs

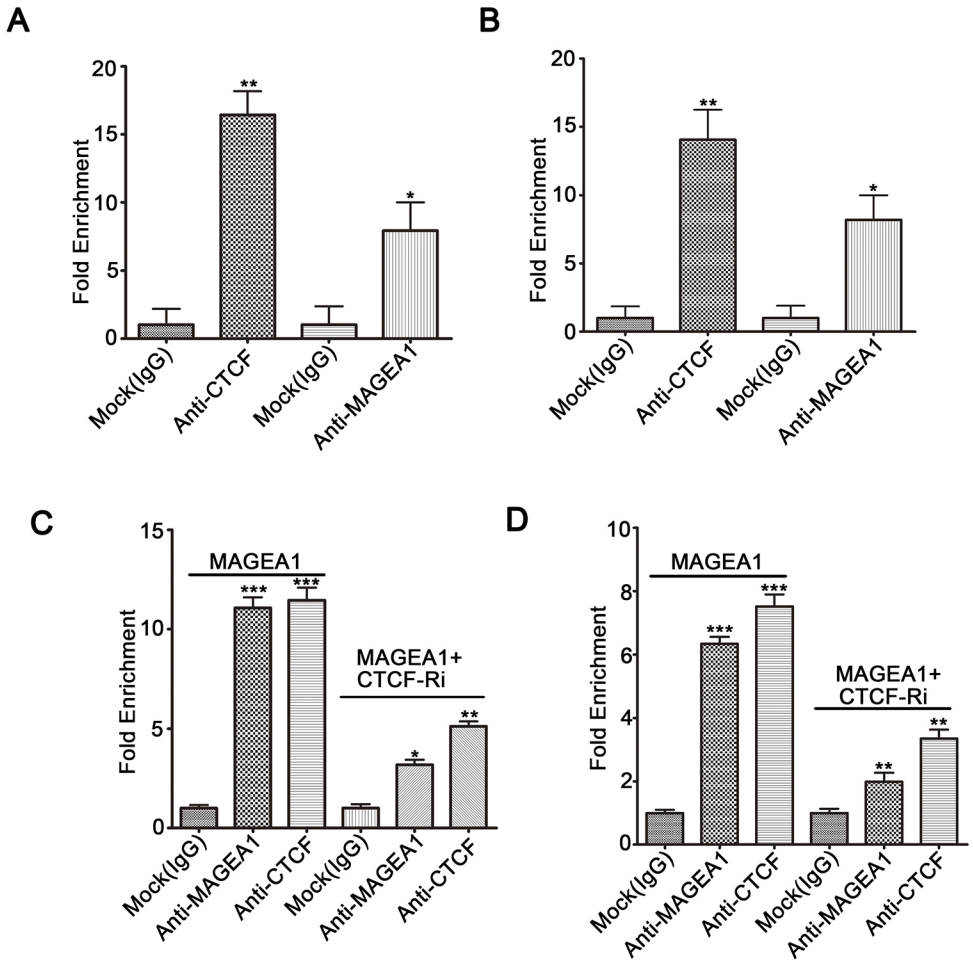

Fig. 5. Downregulation of CTCF reduced MAGEA1 binding to the promoter of BORIS. (A,B) ChIP analysis of MAGEA1 and CTCF binding to the BORIS promoter in MCF-7 (A) and MDA-MB-231 (B) cell lines. (C,D) The effect of CTCF downregulation of MAGEA1 binding to the BORIS promoter analyzed by ChIP in MCF-7 (C) and MDA-MB-231 (D) cells. All experiments were repeated at least three times. **P*<0.05; ***P*<0.01; ****P*<0.001.

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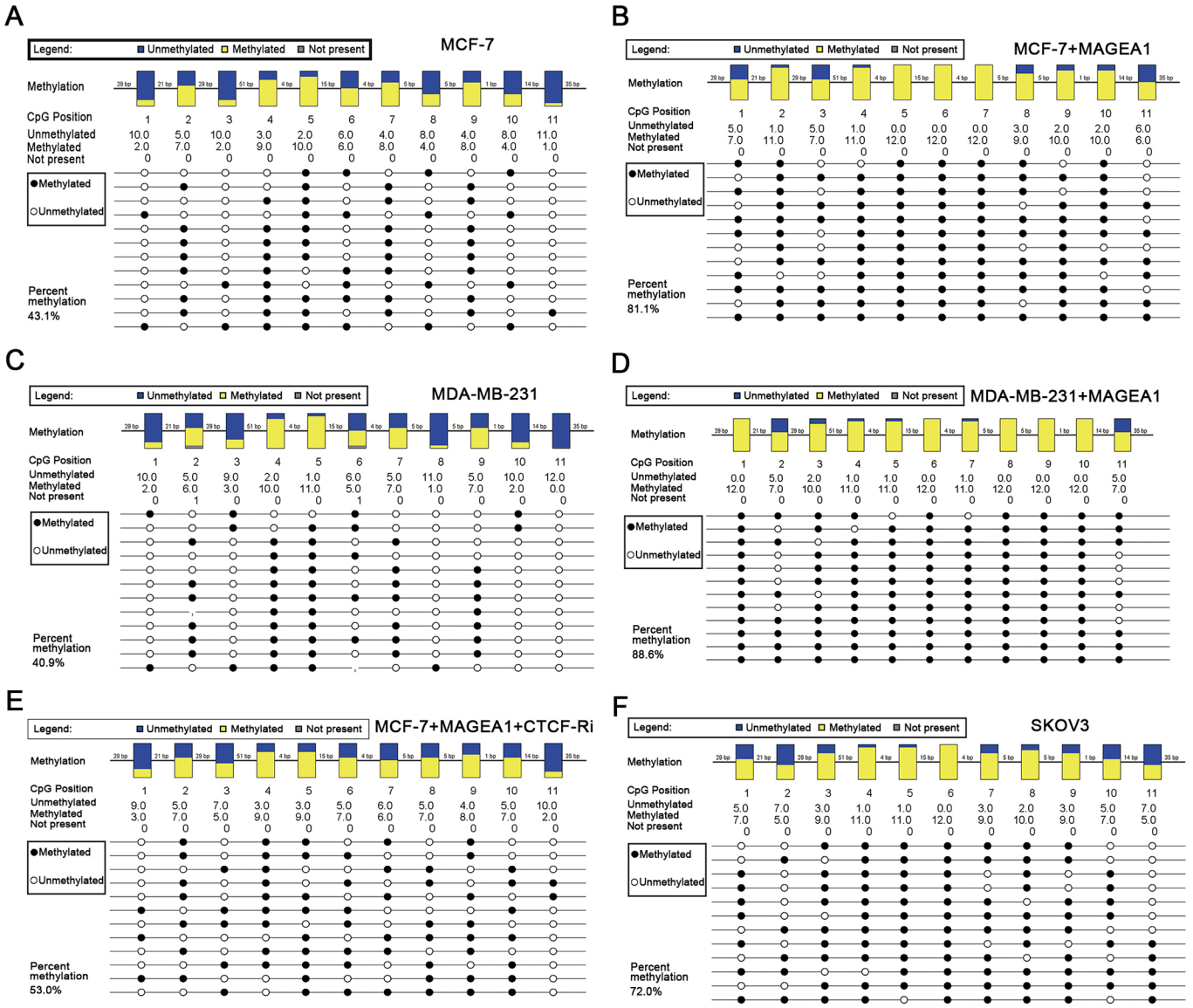

Fig. 6. MAGEA1-induced methylation in the BORIS promoter requires CTCF. (A–D) Genomic bisulfite sequencing of CpGs within the CpG island in MCF-7 (A), MDA-MB-231(C) and their MAGEA1-overexpressing cell lines (B,D). (E) Downregulation of CTCF reduced MAGEA1-induced methlyation in MCF-7 cells. MCF-7+MAGEA1+CTCF-Ri indicates MAGEA1 overexpression but that BORIS was knocked down by siRNA-mediated RNA interference. (F) Endogenous methylation in the BORIS promoter in SKOV3 cells. Open circles (○) and filled circles (●) represent non-methylated CpGs and methylated CpGs, respectively. All experiments were repeated at least three times.

are rather complex and can be cancer type, cell line and/or context dependent (Lim et al., 2012; Zajac et al., 2017). In this study, we have shown that MAGEA1 induces cell death in the breast cancer cell lines tested and revealed the mechanisms involved in depth.

Although it has been shown that BORIS activates MAGEA expression via demethylation in various cell types (Kang et al., 2007; Vatolin et al., 2005), we demonstrate here, for the first time, that MAGEA1 has a negative regulatory effect on BORIS expression, and that downregulation of BORIS is functionally involved in MAGEA1-induced cell death in breast cancer cells. These findings emphasize that different CTAs may play opposing roles in the same or different cells and may counter-regulate each other.

Although CTCF has been reported to inhibit BORIS expression, the molecular mechanisms are not fully understood (Renaud et al., 2007). While we have shown a consistent negative regulatory role of CTCF in BORIS expression in breast cancer cells, new mechanisms have been revealed. We have identified an interaction between MAGEA1 and CTCF, which binds to the BORIS promoter and represses its expression. In addition, we found that CTCF plays crucial roles in MAGEA1-binding capacity to the BORIS promoter and recruitment of DNMT3a to the same complex. Consequentially, downregulation of CTCF reversed the effects of MAGEA1 on BORIS expression and cell death, as well as the interaction between MAGEA1 and DNMT3a. Interestingly, although CTCF is important for the effect of MAGEA1 on BORIS, relatively high levels of CTCF expression in the absence of MAGEA1 expression (such as the endogenous levels in MCF7 and MDA-MB-231 cells; Fig. 3C) are insufficient for induction of hypermethylation of the BORIS promoter (Fig. 6), implying that MAGEA1 is essential to regulate DNMT3a-mediated methylation of the BORIS promoter.

On the other hand, although we have found that DNMT1 downregulation is accompanied by MAGEA1 upregulation and repression of BORIS expression, DNMT1 was not required for

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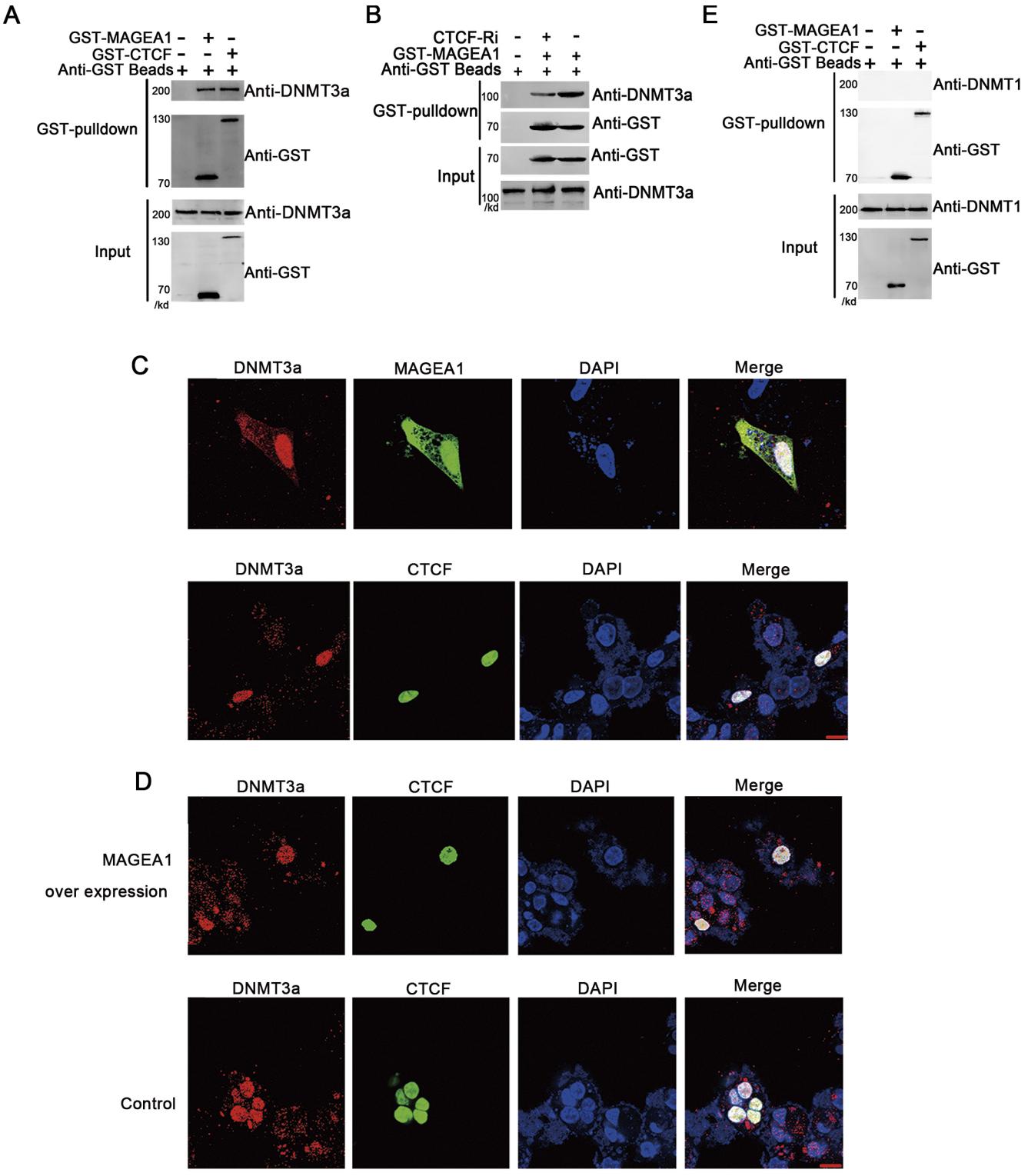

Fig. 7. DNMT3a interacted with MAGEA1 and CTCF. (A) GST pull-down assays performed between DNMT3a and MAGEA1 or CTCF. All of the incubated proteins and antibodies used in the IP and western blot steps are indicated. (B) When CTCF was downregulated (lane 3), DNMT3a co-immunoprecipitation was significantly reduced compared with that in the control (lane 2) MCF-7 cells. (C) The subcellular localizations of MAGEA1 or CTCF and DNMT3a in MCF-7 cells were identified by immunofluorescence. MAGEA1 or CTCF was fused to GFP, showing green fluorescence. DNMT3a was detected by TRITC red fluorescence and nuclei were stained with DAPI (blue fluorescence). (D) Co-localization between CTCF and DNMT3a was detected in MCF-7 control and MAGEA1-overexpressing cells. (E) GST pull-down assay between MAGEA1 or CTCF and DNMT1. Scale bars: 50 µm. All experiments were repeated at least three times.

MAGEA1-induced methylation of the BORIS promoter. These results suggest that a different mechanism is involved in BORIS regulation by DNMT1, which warrants further investigation.

We have identified that DNMT3a, but not another methyltransferase, DNMT1, is responsible for the MAGEA1-induced increased methylation of the BORIS promoter. In addition,

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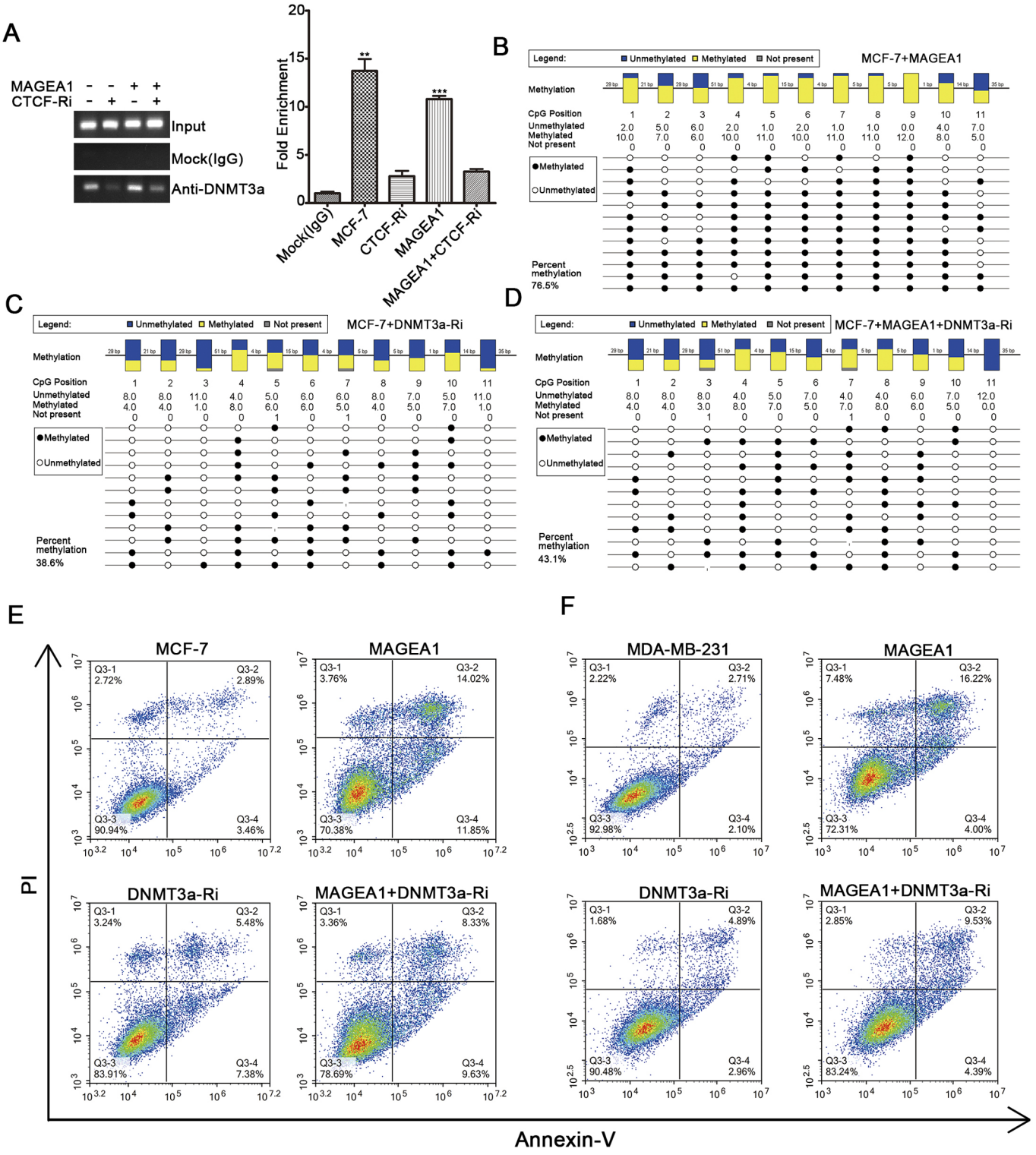

Fig. 8. DNMT3a is functionally involved with MAGEA1. (A) ChIP analysis of DNMT3a binding to the BORIS promoter in MCF-7 cells with MAGEA1 overexpression or CTCF knockdown. (B–D) Methylation levels detected in MAGEA1-overexpressing (B), DNMT3 knockdown (C), and MAGEA1-overexpressing and DNMT3a knockdown (D) MCF-7 cells. (E,F) Annexin V-FITC/PI staining-based flow cytometry detected the apoptosis in MCF-7 (E), MDA-MB-231 (F) parental and their derived cell lines: MAGEA1-overexpressing cells, DNMT3a knockdown cells, and MAGEA1-overexpressing and DNMT3a knockdown cells. All experiments were repeated at least three times. ***P*<0.01; ****P*<0.001.

DNMT3a physically interacts with CTCF and is functionally involved in MAGEA1-induced cell death.

Our results presented here are consistent with reports of BORIS oncogenic activities. As a transcription factor, BORIS activates many oncogenes, including *TSP50*, *TERT*, *OCT4* (also known as *POU5F1*) and *c-Myc* (also known as *MYC*) (Bhan et al., 2011; Liu et al., 2017; Smith et al., 2009; Vatolin et al., 2005; Yang et al., 2015). Selective apoptosis of breast and colorectal cancer cells was observed by

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inhibiting BORIS expression (Dougherty et al., 2008; Zhang et al., 2017). Our annexin V-FITC/PI double-staining assays also show that BORIS has a protective role in MAGEA1-induced cell death.

In conclusion, the current study demonstrates a newly detected tri-complex among MAGEA1, CTCF and DNMT3a. The binding of this complex to the BORIS promoter accounts for the hypermethylation and repression of BORIS expression, which results in subsequent cell death in the breast cancer cell lines tested. Each of the three proteins play interactive, yet distinct, roles in BORIS expression and subsequent cell death. While CTCF is critical for the ability of MAGEA1 to bind to the BORIS promoter, and for the recruitment of DNMT3a, CTCF itself is insufficient to induce hypermethylation in the absence of MAGEA1. On the other hand, MAGEA1 is essential for the induction of hypermethylation of the BORIS promoter, potentially via a direct interaction with DNMT3a to recruit and/or activate DNMT3a, which remains to be further investigated. DNMT3a is likely the enzyme directly catalyzing the methylation, but it relies on both MAGEA1 and CTCF to effectively exert its activity on the BORIS promoter. The mechanistic connections of these proteins are supported by our functional studies conducted. These new findings are likely to have implications in developmental strategies for CTA-based immunotherapeutics.

MATERIALS AND METHODS

Cell lines

Human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection. The ovarian cancer cell line SKOV3 was a generous gift from Prof. Cui (Peking University RenMin Hospital, Beijing, China). All cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal calf serum. Cells were maintained in a humidified incubator at 5% CO_2 and 37°C. The cell lines MCF-7 and MDA-MB-231 have been authenticated and tested for contamination recently.

Plasmids, siRNA and transfection conditions

The entire coding regions of *MAGEA1* and *BORIS* were obtained from complementary DNA prepared from SKOV3 and MCF-7 cells, respectively, by RT-PCR. The primers used for these cloning steps are listed in Table S1. *MAGEA1* was subcloned into the pEGFP-C1 or pHM6-HA plasmid vectors using the *Eco*RI/*Kpn*I and *Hind*III/*Eco*RI sites, respectively. *BORIS* was subcloned into pCMV-14-flag using the *Eco*RI/*Kpn*I sites. The *CTCF* coding region was purchased from the Shanghai Genechem Company (Shanghai, China) and subcloned into pcDNA-3.1-GFP plasmid vectors using the *Eco*RI/*Xba*I sites. For cell-free expression systems, *MAGEA1* and *CTCF* were subcloned into pcDNA-3.1-GST using the *Eco*RI/*Xba*I sites.

The overexpression plasmid vectors were transfected into MCF-7 and SKOV3 cells with Vigofect (Vigorous Biotechnology, Beijing, China), and into MDA-MB-231 cells with Ploylus jetPRIME (Polyplus, Illkirch-Graffenstaden, France), according to the manufacturer's instructions. The gene expression levels were detected by RT-PCR 24 h after transfection, and protein was detected at 36 h with western blot analyses.

For siRNA transfection, subconfluent MCF-7, SKOV3, or MDA-MB-231 cells were transiently transfected using Ploylus jetPRIME with 40 nM non-silencing control siRNA (GenePharma, Shanghai, China) or 100 nM gene-specific targeting siRNAs (GenePharma). The sequences of these siRNAs are listed in Table S2. *DNMT3a* siRNA sequences were the same as those previously reported (Xu et al., 2014). The transfection procedure (including dosage) was performed according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was conducted using a QuantStudio Flex real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) and TransStart Top Green qPCR SuperMix (Transgene Biotech, Beijing, China). The sequences of the primers used are listed in Table S3. The detailed steps and RNA extraction methods were performed as described previously (Zhao et al., 2017).

Western blot analyses

Western blot analyses were performed as described (Zhao et al., 2017). Proteins were detected with antibodies: anti-HA (Abcam, Cambridge, UK), anti-GFP (Roche, Basel, Switzerland), anti-MAGEA1/BORIS and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CTCF (Cell Signaling Technology, Boston, MA), anti-DNMT3a (Abcam), anti-FLAG (Transgene Biotech), goat anti-mouse IgG and goat anti-rabbit IgG (Cwbio, Beijing, China). The batch numbers and dilutions are listed in Table S4.

Co-IP and GST pull-down assays

Cells were lysed in RIPA lysis buffer without sodium dodecyl sulfate (SDS). Cell lysates (1 mg/sample) were incubated with specific antibodies (4 µg) overnight at 4°C. The washed immunoprecipitates were re-suspended in 30 µl of 2× SDS and the proteins were detected by western blot analyses.

For GST pull-down assays in cell-free system, the proteins (GST-MAGEA1, GST-CTCF) were synthesized using the TNT® Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA). The protein (1 mg/sample) in PBS [pH 7.2–7.4 in the presence of with 1% bovine serum albumin (BSA)] was added up to 600 µl (with 1% BSA). Then, the mixed protein solution (to 600 µl) was incubated with specific antibodies overnight at 4°C. After washing with PBS, the bound proteins were detected using western blot analyses.

Flow cytometry analysis for assessment of cell death

Cell death rates were measured by flow cytometry using an annexin V-FITC/PI apoptosis detection kit (Vazyme Biotechnology, Nanjing, China). MCF-7 and MDA-MB-231 cells were collected at a concentration of 1×10^5 cells ml^{-1}, mixed with annexin V-FITC and PI, according to the manufacturer's recommendation, and analyzed using a flow cytometer. Data were analyzed using NovoExpress Software (ACEA Bioscience, San Diego, CA, USA).

Immunofluorescent localization

Cells were cultured on glass coverslips for 24 h in DMEM containing 10% fetal bovine serum. MAGEA1 was tagged by GFP, CTCF was tagged by GFP or tetramethylrhodamine isothiocyanate (TRITC), and DNMT3a was tagged by TRITC. Then, the cells were fixed for 20 min with 3.4% formaldehyde and subsequently treated with 0.5% PBS-Triton X-100 for 10 min. After blocking with 3% BSA in PBS for 30 min, the coverslips were covered with mouse primary antibodies against the tag and incubated at 37°C for 1 h. This was followed by a 1 h incubation with goat anti-mouse IgG tagged with TRITC (ZSGB-BIO Company, Beijing, China). For nuclear staining, 4′,6-diamidino-2-phenylindole (DAPI) was used. The subcellular localization of MAGEA1, CTCF or DNMT3a was detected by fluorescence microscopy.

ChIP assays

MCF-7 and MDA-MB-231 cell lines were used in ChIP assays to examine the cell binding of CTCF, MAGEA1 and DNMT3a to the BORIS promoter. We used a ChIP assay kit (Beyotime Biotechnology, Shanghai, China) and followed the manufacturer's recommendations. One ChIP reaction used 10 µl anti-CTCF or 15 µl anti-DNMT3a/HA-MAGEA1 monoclonal antibody. Immunopurified DNA was used in real-time PCR using the primers listed in Table S5.

Bisulfite genomic sequence

DNA was extracted from culture cells using an EasyPure Genomic DNA Kit (Transgene Biotech). The DNA was modified by sodium bisulfite using a BisulFlash DNA Modification Kit (Epigentek, New York, NY, USA). The experimental operation was carried out in accordance with the instructions. After bisulfite modification, PCR on the BORIS promoter was performed with the primers shown in Table S5. The PCR products were analyzed on 1% agarose gels (Magen Company, Guangzhou, China). The target bands were extracted from the gel. The purified PCR products were cloned into pMD19-T Vector (Takara, Tokyo, Japan). White-blue plaque selection was

performed for positive bacterial strain selection. The products were then sequenced and analyzed by BIQ_Analyzer Microsoft App (Max Planck Institute for Informatics, Saarbrücken, Germany).

Statistical analyses

All experiments were conducted independently at least three times. Statistical analyses were performed using Prism 5 (GraphPad, San Diego, CA, USA) and the data are presented as mean±s.d. *P*-values were calculated by two-sided Student's *t*-test and $P<0.05$ was considered significant. Data were obtained from three independent experiments and presented as average values±s.e.m. (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.Z., Q.L., J.S.; Methodology: J.Z., Y.W., Q.L., J.S.; Software: J.Z., Y.W., Y.X.; Validation: Y.W.; Formal analysis: J.Z., Q.L., Y.X.; Investigation: J.Z., Y.W.; Resources: Q.L., J.S.; Data curation: J.S.; Writing - original draft: J.Z., Y.X.; Writing - review & editing: Y.X.; Project administration: J.S.; Funding acquisition: Y.X., J.S.

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Supplementary information

Supplementary information available online at
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